Redox signaling, Nox5 and vascular remodeling in hypertension

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Purpose of review
Extensive data indicate a role for reactive oxygen species (ROS) and redox signaling in vascular damage in hypertension. However, molecular mechanisms underlying these processes remain unclear, but oxidative post-translational modification of vascular proteins is critical. This review discusses how proteins are oxidatively modified and how redox signaling influences vascular smooth muscle cell growth and vascular remodeling in hypertension. We also highlight Nox5 as a novel vascular ROS-generating oxidase.

Recent findings
Oxidative stress in hypertension leads to oxidative imbalance that affects vascular cell function through redox signaling. Many Nox isoforms produce ROS in the vascular wall, and recent findings show that Nox5 may be important in humans. ROS regulate signaling by numerous processes including cysteine oxidative post-translational modification such as S-nitrosylation, S-glutathionylation and sulfydration. In vascular smooth muscle cells, this influences cellular responses to oxidative stimuli promoting changes from a contractile to a proliferative phenotype.

Summary
In hypertension, Nox-induced ROS production is increased, leading to perturbed redox signaling through oxidative modifications of vascular proteins. This influences mitogenic signaling and cell cycle regulation, leading to altered cell growth and vascular remodeling in hypertension.

Keywords
NADPH oxidase, oxidative post-translational modification, reactive oxygen species, vascular smooth muscle cells

INTRODUCTION
Reactive oxygen species (ROS) are produced in all cell types of the vasculature, including endothelial cells, smooth muscle cells, adventitial fibroblasts and perivascular adipocytes. In the cardiovascular system, the major ROS are superoxide (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl anion (OH⁻). Reactive nitrogen species, including nitric oxide (NO) and peroxynitrite (ONOO⁻), are also biologically important oxidants [1–3]. ROS regulate many cellular processes in the vasculature such as cell growth, contraction/dilation, migration, differentiation and cytoskeletal organization, important in maintaining vascular tone and integrity [4,5**]. In stressed or pathological conditions, ROS-generating enzymes, such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Nox), are activated leading to increased bioavailability of ROS (termed oxidative stress). When produced in excess, ROS interact with lipids and proteins leading to functional and structural changes of target molecules. This perturbation in oxidative balance promotes post-translational oxidative modification of lipids and proteins and impaired redox signaling [6,7]. Post-translational oxidative modification involves covalent changes of cysteine residues within redox-sensitive proteins, important processes that regulate protein structure and function. Redox-sensitive signaling is involved in endothelial dysfunction, arterial remodeling and vascular inflammation associated with hypertension and seems to play an important role in cardiovascular
complications and target organ damage [8,9]. The present review discusses the role of ROS in vascular remodeling in hypertension and focuses on cellular and molecular mechanisms underlying ROS actions. We specifically highlight the potential role of the novel ROS-generating Nox isoform, Nox5 in the vasculature; processes of oxidative post-translational modification and impact on redox signaling and vascular remodeling in hypertension and; targeting Nox/ROS as a potential therapeutic strategy.

**CHARACTERISTICS OF REACTIVE OXYGEN SPECIES IN BIOLOGICAL SYSTEMS**

Most ROS and reactive nitrogen species are relatively unstable oxygen-centered or nitrogen-centered-free radicals, which contain unpaired electrons. Superoxide is water soluble, unstable and short-lived, whereas H$_2$O$_2$ is lipid soluble and more stable than O$_2^*$ [10]. H$_2$O$_2$ is produced mainly from dismutation of O$_2^*$ by superoxide dismutases and is scavenged by catalase and glutathione peroxide [11]. NO is enzymatically formed by nitric oxide synthase, which oxidizes L-arginine. NO acts as a second messenger with vasodilatory, anti-inflammatory and anti proliferative actions. In the presence of excess O$_2^*$, NO is converted into the injurious oxidant, peroxynitrite. ROS are highly reactive and therefore they have short half-lives in biological systems. This makes it very challenging to measure ROS directly and accordingly most assays are indirect estimates of ROS abundance and reactivity.

**PRODUCTION OF REACTIVE OXYGEN SPECIES IN THE VASCULAR WALL**

All cell types of the vascular wall and endothelium have the capacity to produce ROS. Physiologically, ROS are generated in a regulated manner at low concentrations and function as signaling molecules modulating vascular function and structure. In pathophysiological conditions, increased activity/expression of ROS-generating enzymes, and/or decreased activation of antioxidant systems in the vasculature result in increased ROS bioavailability leading to oxidative stress.

ROS are produced as by-products by enzymatic reactions such as those catalyzed by cytochrome P450 enzymes, xanthine oxidase, uncoupling of eNOS, mitochondrial oxidases and glucose oxidase [12–14]. The Nox enzymes are ‘professional oxidases’, and have as their primary function, the production of ROS. They are the major enzymatic source of ROS in the vascular wall. NADPH oxidases are multisubunit enzymes that generate O$_2^*$ by the one electron reduction of oxygen using NADPH as the electron donor (2O$_2$ +NADPH $\rightarrow$ 2O$_2^*$ +NADP$^+$ + H$^+$) [15,16]. The major catalytic subunit is Nox (of which there are seven isoforms, Nox1-5, Duox1 and Duox2), which together with p22phox, are membrane-bound subunits. The cytosolic regulatory subunits include p40phox, p47phox and p67phox or homologues NoxO1 and NoxA1. Noxs are differentially regulated by the subunits and not all Noxs require subunits for activation. In human vascular cells, the major Nox isoforms are Nox1, Nox4 and Nox5. Nox2, the prototype Nox, is found primarily in phagocytic cells and may also be present in the vascular wall in pathological conditions, usually localized in macrophages and invading monocytes. Unlike phagocytic Nox, which is activated only upon stimulation and generates O$_2^*$ in a burst-like manner extracellularly, vascular Nox may be constitutively active, preassembled and produces O$_2^*$ intracellularly in a slow and sustained manner [16].

Vascular Nox is activated by many prohypertensive factors, including vasoactive agents, growth factors, cytokines, shear stress and mechanical forces [17,18]. Among these, angiotensin II (Ang II), through AT$_1$ receptors, appears to be particularly important. Acute stimulation of vascular cells with Ang II causes increased Nox-derived ROS generation leading to activation of redox signaling pathways. Nox expression and activation are increased in cultured endothelial and vascular smooth muscle cells and in whole vessels in experimental and human hypertension [19–21]. Nox hyperactivation leads to excessive ROS generation that disrupts redox networks, normally regulated by antioxidant systems, resulting in oxidative stress, triggering molecular processes, which in the vasculature, contributes to vascular injury.

The biological significance of multiple Nox isoforms being expressed in the vascular wall is unclear,
but their differential tissue distribution, cellular localization and subcellular compartmentalization probably play a major role in Nox-specific actions [22]. For example, whereas Ang II-activated Nox1 appears to be important in vascular smooth muscle cells from large arteries, especially in association with atherosclerosis, Nox4 and Nox5 may be more important in small resistance arteries, especially in humans [23,24]. Moreover, different Nox isoforms may generate different ROS. For example, Nox1, Nox2 and Nox5 generate \( \text{O}_2^- \), whereas Nox4 produces primarily \( \text{H}_2\text{O}_2 \) and may act as a vasodilator in some vascular beds. Nox isoforms in the cardiovascular system have recently been comprehensively reviewed [25–28] and only the most recent of the Nox family members, Nox5, is briefly discussed here.

**VASCULAR NOX5**

Nox5 (five splice variants: \( \alpha, \beta, \delta, \gamma, \varepsilon \)) is the most recently identified Nox and is unique: it is \( \text{Ca}^{2+} \)-sensitive, it possesses a calmodulin-like domain with binding sites for \( \text{Ca}^{2+} \), the binding of which induces a conformational change leading to enhanced ROS formation, and it does not require any NADPH oxidase subunits for its activity [29–31,32**,33–36]. Nox5 phosphorylation of serine/threonine (Ser475, Ser490, Thr494 and Ser498) enhances sensitivity to \( \text{Ca}^{2+} \) and facilitates ROS production at lower levels of \( \text{Ca}^{2+} \) [31]. Nox5 phosphorylation is regulated by protein kinase C (PKC), specifically PKCa. Nox5 is also regulated by other kinases, including c-Abl, \( \text{Ca}^{2+} \)/calmodulin-dependent protein kinase II and MAP kinases [32**,33–35]. Protein:protein interactions with the molecular chaperones Hsp90 and Hsp70 further regulate Nox5 expression and activity [35]. Nox5 was originally discovered in testes, spleen and lymph nodes, but more recently has been found in the vasculature, heart and kidney. In vascular cells, Nox5\( \alpha \) and Nox5\( \beta \) are the major ROS-generating isoforms and are activated by thrombin, platelet-derived growth factor, Ang II and endothelin-1. Although all Noxs are present in mice, rats and humans, the rodent genome does not contain the nox5 gene, making it challenging to study Nox5 in the experimental setting. The biological significance of vascular Nox5 is still unknown, although it has been implicated in cell proliferation, angiogenesis and migration [37–39]. In porcine aortic cells, Nox5-derived ROS is required for growth factor-induced potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4 [40], important in vascular smooth muscle cell proliferation and migration in atherosclerosis [40]. In pathological conditions, such as atherosclerosis, acute myocardial infarction, aneurysm and hypertension, vascular Nox5 expression is increased, implying a role for Nox5 in cardiovascular disease [41,42,43**].

Macrophage and monocyte Nox5 have been shown to play a role in ROS production in atherosclerosis [44]. We recently demonstrated that in mice expressing human Nox5 in a podocyte-specific manner, renal function is markedly impaired and blood pressure is elevated [45], further suggesting a role for Nox5 in disease processes [45].

**REDOX SIGNALING IN VASCULAR CELLS**

ROS are important signaling molecules in vascular smooth muscle cells. In hypertension, perturbations in ROS signaling are associated with endothelial dysfunction, impaired vascular tone and arterial remodeling [46]. These processes are mediated by changes in redox state of ion channels (K\(^+\) channels and Ca\(^{2+}\) channels), cyclases (guanylate cyclase), kinases [mitogen-activated protein kinases (MAPK), Rho kinases and tyrosine kinases], phosphatases (protein tyrosine phosphatases), cytoskeletal proteins (actin and myosin) and activation of transcription factors [activator protein-1 (AP-1), nuclear factor \( \kappa \)B (NF\( \kappa \)B), and nuclear factor erythroid 2-related factor 2] [47–49].

The specific signaling effects of ROS are mediated by the covalent modification of specific cysteine residues in redox-sensitive proteins. These residues have unique features in that they contain a terminal thiol (-SH) functional group, which is electron-rich enabling different oxidation states, including S-nitrosylation (S-nitration; SNO), S-glutathionylation (RS-SG), sulfhydration (SSH), disulfide bonds (RS-RS\(^-\)), sulfinylation (SOH), sulfonic acid (SO\(_2\)H) and sulfonic acid (SO\(_3\)H) [50]. Oxidative post-translational modifications influence target protein structure and function. The multiple types of oxidative post-translational modifications influence target proteins translate into diverse cellular effects, which in vascular cells range from contraction to growth.

**OXIDATIVE POST-TRANSLATIONAL MODIFICATION OF PROTEINS IN THE CARDIOVASCULAR SYSTEM**

**S-Nitrosylation**

SNO of proteins in the cardiovascular system has been associated with protective effects. This has been demonstrated during ischemic preconditioning and has been attributed to SNO of ATP synthase at Cys294 [51]. The post-translational modification
of Cys294 is altered in heart failure, and accordingly this Cys294 in the ATP synthase has been described as a redox switch [51]. In experimental models of hypertension, increased SNO modification of proteins was associated with impaired aortic relaxation [52]. A comprehensive dataset for modified SNO proteins in cardiac, endothelial and vascular smooth muscle cells has been compiled [50,53]. SNO-modified proteins are localized in many subcellular compartments and organelles and are involved in numerous cellular functions, especially those related to cell metabolism and cytoskeletal organization.

**Sulffydration**

H₂S is produced by cystathionine γ-lyase and has been identified as a vasodilator. Mice lacking cystathionine are hypertensive [54]. H₂S can modify downstream proteins by sulffydration (SSH), which is an important Cys modification. H₂S produced in response to endoplasmic reticulum (ER) stress sulffydrates protein tyrosine phosphatase 1B (PTP1B), and possibly other protein tyrosine phosphatases [55]. Sulffydration of PTP1B reduces its activity leading to decreased PTP1B-induced dephosphorylation of protein kinases, such as protein kinase-like ER kinase and MAPK, key proteins that regulate vascular smooth muscle cell function [55].

**S-glutathionylation**

Another form of oxidized post-translational modification of proteins is S-glutathionylation (RS-SG), which is reversible [56,57]. It is formed by a reaction of glutathione (oxidized form, GSSG) or S-nitrosogluthathione (GSNO) with free thiol. Many proteins in cardiovascular cells are able to undergo S-glutathionylation, which when exposed to an oxidative milieu, such as in hypertension and aging, is increased [56,57]. S-glutathionylation of endothelial NOS (eNOS) has recently been identified as an important novel mechanism of eNOS regulation, processes that are altered in cardiovascular disease [58,59].

**Sulfenylation**

Sulfenylation has been associated with injurious oxidative damage. Sulfenic acid is very reactive, unstable and hence is short-lived. Sulfenic acid can be converted into other oxidized post-translational modifications, which may be reversible or irreversible [49].

**OXIDATION OF CYTOSKELETAL PROTEINS IN VASCULAR SMOOTH MUSCLE CELLS**

Of the many proteins that undergo oxidative changes is actin, a major protein involved in cytoskeletal organization in vascular cells [60]. Actin is highly redox-sensitive and when cells are exposed to oxidative stress actin is among the most prominent proteins to become oxidized [60]. Stimulation of vascular cells with H₂O₂ causes cytoskeletal disorganization and morphological changes. In the heart, actin oxidation is associated with impaired contractility [60,61]. In vascular smooth muscle cells, interleukin-22 stimulation leads to carbonylation of actin, as well as α-enolase, heat shock cognate 71kDa protein and mitochondrial 60kDa heat shock protein, proteins associated with cell stress and growth [62].

Growing evidence indicates that oxidized proteins may function collaboratively with proteins that undergo other post-translational modifications such as phosphorylation, acetylation and ubiquitination [49]. This networking between proteins impacts on downstream signaling that determines the final biological cellular response, such as cell growth.

**REDOX REGULATION OF VASCULAR CELL GROWTH: IMPLICATIONS IN HYPERTENSION**

Vascular smooth muscle cells are intrinsically contractile in nature and exhibit very low rates of proliferation. However, in pathological conditions associated with vascular injury, such as in hypertension, vascular smooth muscle cells proliferate, undergo hypertrophy, dedifferentiate and migrate [63]. These processes are regulated in large part through ROS which influence redox-sensitive mitogenic signaling and cell cycle progression through oxidative post-translational modification of proteins, including protein tyrosine phosphatases, protein kinases, cytoskeletal proteins and transcription factors [63–65] (Fig. 1).

Despite both O²⁻ and H₂O₂ inducing vascular smooth muscle cell proliferation, their growth signaling pathways are different. Whereas O²⁻ primarily activates extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase, H₂O₂ increases expression of p38 mitogen-activated protein kinase [66–68]. Mitogenic signaling pathways in vascular smooth muscle cells that are sensitive to oxidant levels also directly influence expression and activity of cyclins and cyclin-dependent kinases, which regulate cell cycle progression [69]. Cdk 4, Cdk 6, Cdk 2 and Cdk 1 are activated by binding with their regulatory subunits cyclin D1, cyclin E, cyclin A and cyclin B, respectively. The kinase activities of cyclin/Cdk complexes are negatively regulated by Cdk inhibitor proteins p21, p27, p16 and p15.
In hypertension, vascular smooth muscle cells are stimulated to divide in response to mitogenic factors. ROS induces variable growth-related responses, including increased proliferation, apoptosis, transient cell cycle arrest, permanent senescence and cell death, depending mainly on the relative dose of exposure. Mitogenic stimulation initiates re-entry into the cell cycle. They exit the G1 phase and enter the S phase. Cyclin D1/Cdk 4, cyclin E/Cdk 2 and cyclin A/Cdk 2 complexes hyperphosphorylate the pRb in the late G1 phase, which then triggers entry into S phase [69,70]. Cyclin D1 controls the G0–G1 transition and is highly sensitive to oxidative stress. It is the only cyclin that can drive terminally differentiated cells back into the cell cycle. Transcription of cyclin D1 is upregulated by growth factors, including Ang II, through ERK1/2 and downstream redox-sensitive transcription factors, such as AP-1 and NFkB, which are highly redox-sensitive [70]. These effects are modulated by microRNA-365 [71].

Oxidants also regulate G1, S and G2 phases of the cell cycle. Peroxides induce a G1 checkpoint response that is attenuated by antioxidants. H2O2 suppresses S phase entry by inhibiting cyclin E/Cdk2 activity, by upregulating p21 and p53 and through downregulation of cyclin A expression [72].

**REACTIVE OXYGEN SPECIES AND VASCULAR REMODELING IN HYPERTENSION**

ROS-induced change of vascular smooth muscle cells to a proliferative phenotype contributes to vascular hypertrophy and remodeling in hypertension, characterized by reduced vascular lumen, increased media thickness, increased stiffness and reduced distensibility [73,74]. At the molecular and cellular levels, remodeling involves changes in cytoskeletal organization, altered growth/apoptosis, senescence and rearrangement of vascular smooth muscle cells, processes that are highly sensitive to alterations in the intracellular redox milieu. Remodeling is also influenced by changes in extracellular matrix protein composition and reorganization of proteoglycans, collagens and fibronectin [74]. Targeting ROS to reduce vascular smooth muscle cell proliferation, prevent vascular cell dedifferentiation and inhibit fibrosis may be an interesting approach to ameliorate arterial remodeling in hypertension [47,75–77].

**FIGURE 1.** Diagram demonstrating mechanisms whereby increased Nox-induced ROS generation regulates redox signaling and vascular smooth muscle cell function in hypertension. Prohypertensive factors, such as activation of the renin angiotensin system, growth factors, sympathetic nervous system activation and proinflammatory mediators, induce activation of Nox isoforms, leading to increased ROS production. Changes in intracellular redox status cause oxidative modification of proteins important in proliferative and profibrotic signaling. Increased vascular smooth muscle cell growth, cytoskeletal reorganization and fibrosis contribute to vascular remodeling in hypertension. ROS, reactive oxygen species.
TARGETING REACTIVE OXYGEN SPECIES AS A THERAPEUTIC STRATEGY IN CARDIOVASCULAR DISEASE

Considering the important role of oxidative damage associated with vascular injury, strategies to reduce ROS bioavailability should have vasoprotective effects in cardiovascular disease. Two major approaches to reduce oxidative stress have been explored including antioxidants to scavenge ROS, and Nox inhibitors to block ROS generation.

Antioxidants

Data from experimental studies have demonstrated that antioxidants improve endothelial function, promote regression of vascular remodeling and reduce blood pressure in hypertension [78,79]. These phenomena are associated with decreased MAP kinase signaling, decreased activation of transcription factors and reduced vascular smooth muscle cell proliferation, inflammation and fibrosis [78–82]. However, clinical findings have been inconsistent and results from antioxidant clinical trials have mainly been negative, showing little cardiovascular benefit of antioxidant vitamins and carotene [83–86]. Possible reasons for these disappointing results from antioxidant trials have been discussed in detail elsewhere [83–86], but a number of points should be highlighted: patients who entered into the trials already had long-standing cardiovascular disease wherein irreversible oxidative damage may have already occurred and hence scavenging of ROS already formed may have little benefit; inappropriate antioxidants may have been used, as antioxidant vitamins themselves can act as oxidants thereby promoting oxidative stress; antioxidant-dosing regimens and duration of therapy may have been suboptimal to effectively scavenge ROS in vivo; orally administered antioxidants may be inaccessible to the source of free radicals, particularly if ROS are generated in intracellular compartments and organelles. This may be especially pertinent to water soluble vitamins, such as vitamin C, which may not cross the cell membrane to scavenge intracellular ROS and; antioxidants do not inhibit the production of ROS; they scavenge free radicals once they are formed. Finally, in none of the large antioxidant clinical trials was it ever proven that patients did indeed have evidence of oxidative stress.

Nox inhibitors

Theoretically, compounds that block ROS generation to reduce an oxidative load should be more efficacious than nonspecific antioxidant ROS scavengers. This is based on experimental evidence in which it has been shown that inhibition of Nox-mediated ROS generation, using pharmacological and gene-targeted strategies, leads to regression of vascular remodeling, improved endothelial function and lowering of blood pressure [87,88]. A number of pharmacological agents have now been developed as NADPH oxidase/Nox inhibitors, including non-specific and isoform-specific compounds.

Classical NADPH oxidase inhibitors, apocynin and diphenyleneiodinium (DPI), are nonspecific and may act as ROS scavengers. Apocynin has intrinsic antioxidant activity and DPI acts as a general flavoprotein inhibitor [76,77,89]. Hence, because of the nonspecific nature of these agents, they should not be used as selective Nox inhibitors.

Recently, new isoform-specific Nox inhibitors have been characterized from rational drug delivery. These include the small molecule inhibitors GKT137831 and GKT136901 (GenKyoTex), which variably inhibit Nox1, Nox4 and Nox5; 2-actylphenothiazine (ML171) (Scripps Research Institute), which inhibits Nox1; VAS2870 and VAS3947 (Vasopharm GmbH), which inhibit mainly Nox2 and to a lesser extent Nox4; S17834 (Servier), which inhibits Nox2 and Nox4; and Fulvene-5, which inhibits Nox2 and Nox4 [75–77]. Biological peptidic inhibitors of NADPH oxidase have been developed by the Pagano group [90**,91], including NOX2ds-tat, which prevents p47phox binding to Nox2, thereby preventing assembly of the active Nox2 oxidase, and NOX1ds, a Nox1 inhibitor, which inhibits binding of NOXA1 to NOX1.

Of the Nox inhibitors that have been registered in the patent literature [76,77,89,90**], only one has progressed through to clinical trials, specifically GKT137831, which has entered into a phase 2 trial in diabetic nephropathy (www.genkyotex.com). Clinical outcomes of this trial should shed light on the role of Nox as a therapeutic target in oxidative stress-related diseases. Further clinical studies are still needed to confirm the clinical utility of Nox inhibitors, but these drugs may hold some promise in patients with Nox/ROS-associated diseases.

CONCLUSION

In hypertension, dysregulation of ROS-generating enzymes, including the novel NADPH oxidase, Nox5, results in oxidative stress, which contributes to vascular damage, through multiple processes including activation of transcription factors, stimulation of mitogenic signaling pathways and modulation of cell cycle progression. Fundamental to these phenomena is cysteine oxidative post-translational modification of vascular proteins, which
determines final cellular responses to oxidative stimuli. The field of oxidative proteomics [92] in the vascular system is still immature and the vascular oxidative proteome in hypertension has yet to be elucidated, but increasing evidence indicates that oxidation of vital cytoskeletal proteins, kinases and phosphatases in vascular smooth muscle cells is critical in phenotype switches in pathological conditions. Although inconclusive at present, strategies to regulate ROS bioavailability by decreasing production and/or by increasing radical scavenging may regress vascular remodeling, prevent vascular damage and reduce hypertension and its associated end-organ damage. Targeting oxidative stress with novel Nox inhibitors and other ROS modulators may be an attractive therapeutic strategy to ameliorate endothelial dysfunction and vascular damage in hypertension and associated diseases. Outcomes of current clinical studies evaluating cardiovascular and renal effects of Nox inhibitors will shed light on the clinical utility of this approach and will also inform on the role of Nox/ROS in human disease.

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Conflicts of interest
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REFERENCES AND RECOMMENDED READING
Papers of particular interest, published within the annual period of review, have been highlighted as:
* of special interest
** of outstanding interest

33. This study advances the field of Nox5 biology by demonstrating mechanisms whereby kinases, specifically PKC, regulate activity of Nox5.


